BARD Final Scientific Report

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Project Title: **The Role of Reactive Oxygen Species (ROS) in the Tritrophic Interactions in Postharvest Biocontrol Systems**

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Keywords *not* appearing in the title and in order of importance. Avoid abbreviations.

Yeast antagonists, mode of action, microarray, induction of resistance, stress tolerence

Abbreviations commonly used in the report, in alphabetical order**:**

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Signature
Principal Investigator

Signature
Authorizing Official, Principal Institution

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Dr. Vera Hershokovitz
Jia Liu

Cooperation Summary (numbers)

	From US to Israel	From Israel to US	Together, elsewhere	Total
Short Visits & Meetings	1	1		2
Longer Visits (Sabbaticals)				

Description of Cooperation:

The research conducted during the whole duration of the project was carried out in full cooperation between the two Israeli and the American laboratories. S. Droby (ARO) and M. Wisniewski (USDA-ARS, Appalachian Fruit Research Lab) met three times: First meeting took place during a workshop held in the US on October 2010 followed by two meetings during 2011 and 2012. Numerous discussions via the e-mail and phone were done to exchange protocols, explain results and plan experiments. Plant material was exchanged by the two teams and used for experiments and analysis according to the expertise of each laboratory. six manuscripts were already published. Additional one in review and one in preparation. Results were jointly presented in a number of national and International conferences.

Patent Summary (numbers)

	Israeli inventor (s) only	US inventor (s) only	Joint IS/US inventors	Total
Submitted	--	--	--	--
Issued (allowed)	--	--	--	---
Licensed	--	--	--	--

Table of Contents

Page

Abstract …….5

Achievements ……...6

Research Objectives of the Research proposal ……6

Unpublished Data…….11

List of Publications ……..…… …...12

Reprints, manuscripts and other publications ……... ..13

Abstract

To elucidate the role of ROS in the tri-trophic interactions in postharvest biocontrol systems a detailed molecular and biochemical investigation was undertaken. The application of the yeast biocontrol agent *Metschnikowia fructicola*, microarray analysis was performed on grapefruit surface wounds using an Affymetrix Citrus GeneChip. the data indicated that 1007 putative unigenes showed significant expression changes following wounding and yeast application relative to wounded controls. The expression of the genes encoding Respiratory burst oxidase (*Rbo*), mitogen-activated protein kinase (*MAPK*) and mitogen-activated protein kinase kinase (*MAPKK*), G-proteins, chitinase (*CHI*), phenylalanine ammonia-lyase (*PAL*), chalcone synthase (*CHS*) and 4-coumarate-CoA ligase (*4CL*). In contrast, three genes, *peroxidase* (*POD*), *superoxide dismutase* (*SOD*) and *catalase* (*CAT*), were down-regulated in grapefruit peel tissue treated with yeast cells.
The yeast antagonists, *Metschnikowia fructicola* (strain 277) and *Candida oleophila* (strain 182) generate relatively high levels of super oxide anion (O2−) following its interaction with wounded fruit surface. Using laser scanning confocal microscopy we observed that the application of M. fructicola and *C. oleophila* into citrus and apple fruit wounds correlated with an increase in H2O2 accumulation in host tissue. The present data, together with our earlier discovery of the importance of H_2O_2 production in the defense response of citrus flavedo to postharvest pathogens, indicate that the yeast-induced oxidative response in fruit exocarp may be associated with the ability of specific yeast species to serve as biocontrol agents for the management of postharvest diseases.
Effect of ROS on yeast cells was also studied. Pretreatment of the yeast, *Candida oleophila*, with 5 mM H_2O_2 for 30 min (sublethal) increased yeast tolerance to subsequent lethal levels of oxidative stress (50 mM H_2O_2), high temperature (40 °C), and low pH (pH 4). Suppression subtractive hybridization analysis was used to identify genes expressed in yeast in response to sublethal oxidative stress. Transcript levels were confirmed using semi quantitative reverse transcription-PCR. Seven antioxidant genes were up regulated. Pretreatment of the yeast antagonist *Candida oleophila* with glycine betaine (GB) increases oxidative stress tolerance in the microenvironment of apple wounds. ROS production is greater when yeast antagonists used as biocontrol agents are applied in the wounds. Compared to untreated control yeast cells, GB-treated cells recovered from the oxidative stress environment of apple wounds exhibited less accumulation of ROS and lower levels of oxidative damage to cellular proteins and lipids. Additionally, GB-treated yeast exhibited greater biocontrol activity against Penicillium expansum and *Botrytis cinerea*, and faster growth in wounds of apple fruits compared to untreated yeast. The expression of major antioxidant genes, including peroxisomal catalase, peroxiredoxin TSA1, and glutathione peroxidase was elevated in the yeast by GB treatment.
A mild heat shock (HS) pretreatment (30 min at 40 1C) improved the tolerance of *M. fructicola* to subsequent high temperature (45 1C, 20–30 min) and oxidative stress (0.4 mol^{-1}) hydrogen peroxide, 20–60 min). HS-treated yeast cells showed less accumulation of reactive oxygen species (ROS) than non-treated cells in response to both stresses. Additionally, HS-treated yeast exhibited significantly greater (P≥0.0001) biocontrol activity against *Penicillium expansum* and a significantly faster (Po0.0001) growth rate in wounds of apple fruits stored at 25 1C compared with the performance of untreated yeast cells. Transcription of a trehalose-6-phosphate synthase gene (TPS1) was up regulated in response to HS and trehalose content also increased.

We performed a transcriptome analysis of the yeast *M. fructicola* using next-generation sequencing technology for RNA (RNA-Seq), to examine the tri-trophic response of *M. fructicola* with citrus fruit and the postharvest pathogen, *Penicillium digitatum*. More than 26 million sequencing reads were assembled into 9,674 unigenes. Approximately 50% of the unigenes could be annotated based on homology matches in the NCBI database. These results provided additional support for the differential expression identified in the transcriptomic analysis using RNA-Seq .This study provides new insight into the biology of the tritrophic interactions that occur in a biocontrol system such as the use of the yeast, M. fructicola for the control of green mold on citrus caused by *P. digitatum*.

Achievements:

1. Molecular changes taking place in fruit tissue following the application of yeast biocontrol agents was characterized.

2. Host Genes associated with induction of resistance in general and MAPK pathway were studied and characterized.

3. Evidence for ROS production and kinetics in fruit tissue following the application of yeast antagonists provided.

4. Effect of elevated levels on yeast biocontrol activity and the ability of the pathogen to infect the tissue were studied. Pretreatment of yeast cells with sublethal concentrations of H_2O_2 increased its tolerance to oxidative stress and heat.

5. Global genes expression in yeast cells following its interaction with either fruit tissue of pathogen's mycelium was characterized.

6. Production superoxids anions by the yeast antagonist *Metschnikowia fructicola* and its involvement in its mode of action was characterized.

Research Objectives as presented in the research proposal:

1. Characterize the kinetics of host ROS production and changes in the expression of genes related to ROS production or scavenging in fruit tissue as a response to yeast antagonists cells and exogenous ROS.

2. Characterize the effect of ROS on host genes associated with MAPK signaling cascade leading to host defense reactions.

3. Determine the implications of elevated ROS production (induced by antagonists yeasts or other factors) at the infection sites (surface wounds) on the ability of pathogenic fungi to infect host tissue.

4. Examine the effects of ROS on the tolerance of yeast antagonists to stress conditions (dehydration, nutrient starvation, osmotic stress).

1. Characterize the kinetics of host ROS production and changes in the expression of genes related to ROS production or scavenging in fruit tissue as a response to yeast antagonists cells and exogenous ROS

To gain a better understanding of the molecular changes taking place in citrus fruit tissue following the application of the yeast biocontrol agent *Metschnikowia fructicola*, microarray analysis was performed on grapefruit surface wounds using an Affymetrix Citrus GeneChip. Using a cut-off of $P < 0.05$ and a 1.5-fold change difference as biologically significant, the data indicated that 1007 putative unigenes showed significant expression changes following wounding and yeast application relative to wounded controls. Microarray results of selected genes were validated by reverse transcription-quantitative real-time polymerase chain reaction (RT-qPCR). The data indicated that yeast application induced the expression of the genes encoding Respiratory burst oxidase (*Rbo*), mitogen-activated protein kinase (*MAPK*) and mitogen-activated protein kinase kinase (*MAPKK*), G-proteins, chitinase (*CHI*), phenylalanine ammonia-lyase (*PAL*), chalcone synthase (*CHS*) and 4-coumarate-CoA ligase (*4CL*). In contrast, three genes, *peroxidase* (*POD*), *superoxide dismutase* (*SOD*) and *catalase* (*CAT*), were down-regulated in grapefruit peel tissue treated with yeast cells. Moreover, suppression was correlated with significantly higher levels of hydrogen peroxide, superoxide anion and hydroxyl radical production in yeast-treated surface wounds. Interestingly, large amounts of hydrogen peroxide were detected inside yeast cells recovered from wounded fruit tissue, indicating the ability of the yeast to activate reactive oxygen species when it is in contact with plant tissue. This study provides the first global picture of gene expression changes in grapefruit in response to the yeast antagonist *M. fructicola*.
The application of *M. fructicola* to discs of grapefruit peel resulted in increased levels of H2O2 in fruit discs. relative to water control peel discs, with a maximum value at 5 h after yeast application. Superoxide anion and hydroxyl radical, however, displayed different trends compared with H_2O_2. Significantly higher levels of superoxide anion and hydroxyl radical in response to yeast antagonist were found after 5 h of application relative to water-treated discs. These levels continued to increase during 24 and 48 h following application relative to the basal levels in control discs

This work was publication in "Molecular Plant Pathology" (see List of publications for more details).

2. Characterize the effect of ROS on host genes associated with MAPK signaling cascade leading to host defense reactions.

Findings of the microarray analysis demonstrated the up-regulation of various genes involved in the MAPK signaling cascade. To further characterize key genes in this signaling pathway, RT-qPCR analysis was carried out. The time points included in the analysis were 2, 8, 24 and 48 h after yeast treatment. The MAPKK expression showed significant increase in transcript levels after 24 and 48 h in yeast-treated wounds relative to the non-treated control. *Metschnikowia fructicola* application also resulted in higher MAPK expression levels at 8, 24 and 48 h relative to controls.

Results included in the article published in "Molecular Plant Pathology" (see List of publications for more details).

3. Determine the implications of elevated ROS production (induced by antagonists yeasts or other factors) at the infection sites (surface wounds) on the ability of pathogenic fungi to infect host tissue.

Metschnikowia fructicola applied to grapefruit wounds exhibited an intense fluorescence, indicating a high level of intracellular H2O2, 5 h following the application of yeast to grapefruit wounds; however, this intensity decreased dramatically after 24 h and remained low at 48 h. Quantification of the fluorescence signal showed that yeast cells reached their highest level of fluorescence 5 h after application to the fruit and decreased after 24 and 48 h.. In contrast, yeast cells grown in nutrient yeast dextrose broth (NYDB) displayed elevated accumulation of H_2O_2 only after 48 h.

A pretreatment of the yeast, *Candida oleophila*, with 5 mM H_2O_2 for 30 min (sublethal) increased yeast tolerance to subsequent lethal levels of oxidative stress (50 mM H2O2), high temperature (40 °C), and low pH (pH 4). Compared with non-stress-adapted yeast cells, stress-adapted cells exhibited better control of apple fruit infections by *Penicillium expansum* and *Botrytis cinerea* and had initially higher growth rates in apple wounds. Suppression subtractive hybridization analysis was used to identify genes expressed in yeast in response to sublethal oxidative stress. Transcript levels were confirmed using semi quantitative reverse transcription-PCR. Seven antioxidant genes were up regulated. The elevated expression of these genes was associated with less accumulation of reactive oxygen species and a lower level of protein and lipid oxidation under subsequent stresses. These data support the premise that induction of abiotic stress tolerance in biocontrol yeast can improve biocontrol efficacy by up regulation of genes involved in the amelioration of oxidative stress.

Results of this work were published in "FEMS Microbiology Ecology". (see List of publications for more details).

Pretreatment of the yeast antagonist *Candida oleophila* with glycine betaine increases oxidative stress tolerance in the microenvironment of apple wounds. ROS production is greater when yeast antagonists used as biocontrol agents are applied in the wounds. These

phenomena result in an oxidative stress environment for the yeast antagonists. It has been demonstrated that pre-exposure of some of these yeast ntagonists to sublethal abiotic stress (heat or hydrogen peroxide), or stress-ameliorating compounds such as glycine betaine (GB) can induce subsequent oxidative stress tolerance in the antagonistic yeast. The increased level of oxidative stress tolerance has been demonstrated in vitro and is characterized by higher levels of antioxidant gene expression, increased production of trehalose, and lower levels of ROS when yeast are exposed to a subsequent oxidative stress. The current study determined whether or not the effects of GB on yeast antagonists determined in vitro persist and are present in planta when yeast are applied to wounded apples. The effect of exogenous GB on the production of ROS in the yeast antagonist, *Candida oleophila*, was determined after the yeast was placed in apple wounds. Oxidative damage to yeast cells recovered from apple wounds was also monitored. Results indicated that GB treatment improved the adaptation of C. oleophila to apple fruit wounds. Compared to untreated control yeast cells, GB-treated cells recovered from the oxidative stress environment of apple wounds exhibited less accumulation of ROS and lower levels of oxidative damage to cellular proteins and lipids. Additionally, GB-treated yeast exhibited greater biocontrol activity against Penicillium expansum and Botrytis cinerea, and faster growth in wounds of apple fruits compared to untreated yeast. The expression of major antioxidant genes, including peroxisomal catalase, peroxiredoxin TSA1, and glutathione peroxidase was elevated in the yeast by GB treatment. This study supports the premise that activation of antioxidant response in biocontrol yeast can improve biocontrol efficacy.

Results of this work were published in "International Journal of Food Microbiology". (see List of publications for more details).

The effect of H_2O_2-induced oxidative stress on the viability of the yeast antagonist, *Cystofilobasidium infirmominiatum*, as well as the effect of exogenous glycine betaine (GB) on yeast viability under oxidative stress, was determined. GB treatment improved the tolerance of *C. infirmominiatum* to oxidative stress. Compared to untreated control yeast cells, GB-treated cells showed less accumulation of reactive oxygen species (ROS) and a lower level of protein oxidation in response to oxidative stress. Additionally, GB-treated yeast exhibited greater biocontrol activity against *Penicillium expansum* and a faster growth in wounds of apple fruits stored at 25 °C compared to the performance of untreated yeast. The activities of antioxidant enzymes, including catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPX) of *C. infirmominiatum* were elevated by GB treatment. Results indicate that the elicitation of antioxidant response by GB may contribute to improvements in oxidative stress tolerance, population growth in apple wounds, and biocontrol activity of *C. infirmominiatum*.

Results of this work were published in "International Journal of Food Microbiology". (see List of publications for more details).

The effect of high temperature and oxidative stress on the cell viability of the yeast antagonist, *Metschnikowia fructicola* was determined. A mild heat shock (HS) pretreatment (30 min at 40 1C) improved the tolerance of *M. fructicola* to subsequent high temperature (45 1C, 20–30 min) and oxidative stress (0.4 mol-^{1}) hydrogen peroxide, 20–60 min). HS-treated yeast cells showed less accumulation of reactive oxygen species (ROS) than non-treated cells in response to both stresses. Additionally, HS-treated yeast exhibited significantly greater (Po0.0001) biocontrol activity against *Penicillium expansum* and a significantly faster (Po0.0001) growth rate in wounds of apple fruits stored at 25 1C compared with the performance of untreated yeast cells. Transcription of a trehalose-6-phosphate synthase gene (TPS1) was upregulated in response to HS and trehalose content also increased. Results indicate that the higher levels of trehalose induced by the HS may contribute to an improvement in ROS scavenging, stress tolerance, population growth in apple wounds and biocontrol activity of M. fructicola.

Results of this work were published in "FEMS Microbiology Ecology". (see List of publications for more details).

The yeast antagonists, *Metschnikowia fructicola* (strain 277) and *Candida oleophila* (strain 182) generate greater levels of super oxide anion (O2−) on intact fruit surfaces (poor in nutrients) then those applied on a nutrient-poor agar medium. Even though yeast antagonists show a high level of O2− on nutrient-rich media, when applied on fruits around wounds (areas abundant in nutrients) accumulation ofO2−, as detected by nitro blue tetrazolium staining, occurred much more rapidly on the latter. Using laser scanning confocal microscopy we observed that the application of M. fructicola and *C. oleophila* into citrus and apple fruit wounds correlated with an increase in H2O2 accumulation in host tissue. In citrus fruit, the level of H_2O_2 around inoculated wounds increased by 4-fold compared to controls (wounds inoculated with water) as early as 18 h after inoculation. Yeast continued to stimulate H_2O_2 production in citrus fruit up to 66 h after inoculation and H_2O_2 levels were still 3-fold above the control. Living yeast cells were detected in fruit wounds at this time point indicating the ability of *M. fructicola* to tolerate host ROS, which has been reported to be an intrinsic characteristic of efficient yeast antagonists. Similar increase in H_2O_2 accumulation around yeast-inoculated wounds was observed in apple fruit exocarp. The present data, together with our earlier discovery of the importance of H_2O_2 production in the defense response of citrus flavedo to postharvest pathogens, indicate that the yeast-induced oxidative response in fruit exocarp may be associated with the ability of specific yeast species to serve as biocontrol agents for the management of postharvest diseases.

Results of this work were published in "Postharvest Biology and technology". (see List of publications for more details).

Description of non published data

De-novo assembly and Characterization of the Transcriptome of Metschnikowia fructicola reveals differences in gene expression following interaction with Penicillium digitatum and grapefruit peel

We performed a transcriptome analysis, using next-generation sequencing technology for RNA (RNA-Seq), to examine the tri-trophic response of *M. fructicola* with citrus fruit and the postharvest pathogen, *Penicillium digitatum*. More than 26 million sequencing reads were assembled into 9,674 unigenes. Approximately 50% of the unigenes could be annotated based on homology matches in the NCBI database. Based on homology, sequences were annotated with a gene description, gene ontology (GO term), and clustered into functional groups. An analysis of differential expression when the yeast was interacting with the fruit vs. the pathogen revealed more than 250 genes with specific expression responses. In the antagonist-pathogen interaction, genes related to transmembrane, multidrug transport and to amino acid metabolism were induced. In the antagonist-fruit interaction, expression of genes involved in oxidative stress, iron homeostasis, zinc homeostasis, and lipid metabolism were induced. Patterns of gene expression in the two interactions were examined at the individual transcript level by quantitative real-time PCR analysis (RT-qPCR). These results provided additional support for the differential expression identified in the transcriptomic analysis using RNA-Seq .This study provides new insight into the biology of the tritrophic interactions that occur in a biocontrol system such as the use of the yeast, M. fructicola for the control of green mold on citrus caused by *P. digitatum*.

Results of this work was accepted for publication in "BMC Genomics". (see attached manuscript).

List of Publications:

Macarisin, D., Droby, S., Bauchan, G. and Wisniewski, M. (2010). Superoxide anion and hydrogen peroxide in the yeast antagonist–fruit interaction: A new role for reactive oxygen species in postharvest biocontrol. *Postharvest Biol. Technol.* 58:194–202.

Liu, J., Wisniewski, M., Droby, S**.**, Tian, S., and Hershkovitz, V. (2011). Effect of heat shock treatment on stress tolerance and biocontrol efficacy of *Metschnikowia fructicola*. *FEMS Microbiol. Ecol.* 76:145-155.

Liu, J., Wisniewski, M., **Droby, S.**, Vero, S., Tian, S., and Hershkovitz, V. (2011). Glycine betaine improves oxidative stress tolerance and biocontrol efficacy of antagonistic yeast *Cystofilobasidium infirmominiatum*. *Int. J. Food Microbiol.* 146:76-83.

Hershkovitz, V., Ben-Dayan, C., Raphael, G., Pasmanik-Chor, M., Liu, J., Belausov, E., Aly, R., Wisniewski, M., and Droby, S. (2012). Global changes in gene expression of grapefruit peel tissue in response to the yeast biocontrol agent *Metschnikowia fructicola*. *Mol. Plant Pathol*. 13(4), 338–349.

Jia, L., Wisniewski, M., Droby, S., Norelli, J., Hershkovitz, V., Tian, S., and Farrell, R.[T] (2012) Increase in antioxidant gene transcripts, stress tolerance and biocontrol efficacy of Candida oleophila following sublethal oxidative stress exposure. *FEMS Microbiol. Ecol*. 80: 578–590.

Sui, Y , Liu, J. , Wisniewski, M., Droby, S. , Norelli, J. , Hershkovitz, V. (2012) Pretreatment of the yeast antagonist, Candida oleophila, with glycine betaine increases oxidative stress tolerance in the microenvironment of apple wounds. *International Journal of Food Microbiology* 157:45-51

Hershkovitz, V., Sela, N., Taha, L., Levy, M., Liu, L., Rafael, G., Kessler, C., Wisniewski, M., Aly, R. and Droby, S. (2012) De-novo Assembly and Characterization of the Transcriptome of Metschnikowia fructicola Reveals Differences in Gene Expression following Interaction with Penicillium digitatum and Grapefruit Peel. *BMC Genomics* (Accepted).

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